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# *Cryptosporidium* spp. in pet birds: Genetic diversity and potential public health significance

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ABSTRACT

To characterize the prevalence and assess the zoonotic transmission burden of *Cryptosporidium* species/genotypes in pet birds in Henan, China, 434 fecal samples were acquired from 14 families of birds in pet shops. The overall prevalence of *Cryptopsoridium* was 8.1% (35/434) by the Sheather's sugar flotation technique. The *Cryptosporidium*-positive samples were analyzed by DNA sequence analysis of the small subunit (SSU) rRNA gene. Three *Cryptosporidium* species and two genotypes were identified, including *C. baileyi* (18/35 or 51.4%) in five red-billed leiothrixes (*Leiothrix lutea*), four white Java sparrows (*Padda oryzivora*), four common mynas (*Acridotheres tristis*), two zebra finches (*Taeniopygia guttata*), a crested Lark (*Galerida cristata*), a Gouldian finch (*Chloebia gouldiae*), and a black-billed magpie (*Pica pica*); *Cryptosporidium meleagridis* (3/35 or 8.6%) in a Bohemian waxwing (*Bombycilla garrulus*), a Rufous turtle dove (*Streptopelia orientalis*), and a fan-tailed pigeon (*Columba livia*); *Cryptosporidium galli* (5/35 or 14.3%) in four Bohemian waxwings (*Bombycilla garrulus*) and a silver-eared Mesia (*Leiothrix argentauris*); *Cryptosporidium* avian genotype III (3/35 or 8.6%) in two cockatiels (*Nymphicus hollandicus*) and a red-billed blue magpie (*Urocissa erythrorhyncha*); and *Cryptosporidium* avian genotype V (6/35 or 17.1%) in six cockatiels (*Nymphicus hollandicus*). Among the pet birds, 12 species represented new hosts for *Cryptosporidium* infections. The presence of *C. meleagridis* raises questions on potential zoonotic transmission of cryptosporidiosis from pet birds to humans.



## 1. Introduction

Cryptosporidiosis is a major protozoan disease in birds and has been reported in more than 30 avian species worldwide (Ryan, 2010). Although only three avian *Cryptosporidium* spp. are recognized, including *Cryptosporidium meleagridis*, *Cryptosporidium baileyi*, and *Cryptosporidium galli* (Slavin, 1955; Current et al., 1986; Ryan et al., 2003), recent molecular epidemiologic studies have improved the understanding of the genetic diversity of *Cryptosporidium* spp. in birds. A number of genetically distinct avian genotypes have been described, including the avian genotypes (I–V), the goose genotypes (I–IV), the black duck genotype, and the Eurasian woodcock genotype (Morgan et al., 2001; Xiao et al., 2002; Jellison et al., 2004; Zhou et al., 2004; Meireles et al., 2006; Ng et al., 2006; Abe and Makino, 2010). These genotypes may be renamed as distinct species in the future once more biological data become available. In addition, *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium serpentis*, *Cryptosporidium muris* and *Cryptosporidium andersoni* were also identified by PCR in a small number of birds, most of which were probably the results of accidental ingestion of oocysts of these organisms (Ryan et al., 2003; Zhou et al., 2004; Ng et al., 2006).

In China, studies on *Cryptosporidium* infection in birds were mainly concentrated in poultry. Two *Cryptosporidium* species *C. baileyi* and *C. meleagridis* have been identified in chickens and quail (Zhang et al., 2004; Wang et al., 2007). In a more recent study, *C. baileyi* was identified in five Ruddy Shelducks (*Tadorna ferruginea*) by microscopical and molecular analyses (Amer et al., 2010). Thus, the distribution of *Cryptosporidium* species/genotypes in birds in China is still unclear. China has one of the largest number of bird species in the world, with at least 1332 species (Zheng, 2005). The breeding of pet birds started in the Zhou Dynasty (from the mid-11th century BC to 256 BC), and about 100 species of birds are kept as pet birds in the present (Zheng, 2005). The aim of this study was to examine the prevalence and identity of *Cryptosporidium* spp. in pet birds in Henan, China.

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## 2. Materials and methods

### 2.1. Sample collection and examination

From March 2008 to April 2009, a total of 434 fresh fecal samples were obtained from pet birds belonging to 32 species from 14 families (Psittacidae, Sturnidae, Alaudidae, Ploceidea, Bombycillidae, Timaliidae, Zosteropidae, Fringillidae, Paridae, Corvidae, Phasianidae, Muscicapidae, Columbidae, and Cuculidae). These samples were collected from pet shops in Zhengzhou City, Henan Province, and fecal material from birds in one cage (1–20 birds, with the same speices) was considered as a sample. Stool samples were examined for *Cryptosporidium* oocysts by bright field microscopy under 400× after oocysts were concentrated by the Sheather's sugar flotation technique. 100 *Cryptosporidium* oocysts from one isolate representing each species/genotype were measured using a Nikon light microscope (model ECLIPSE E200) under a magnification of ×1000. *Cyptosporidium*-positive samples were stored in 2.5% potassium dichromate at 4 °C.

### 2.2. DNA extraction

*Cryptosporidium* oocysts were isolated from the positive fecal samples by the discontinuous density sucrose gradient centrifugation. Genomic DNA was extracted from the purified oocysts using the Mag Extractor-Genome kit (Toyobo Co. Ltd., Osaka, Japan), based on chaotropic extraction followed by absorption of DNA onto silica-coated magnetic beads, using the previous described procedures (Wang et al., 2008). The eluted DNA was kept at −20 °C before it was used in molecular analysis.

### 2.3. Cryptosporidium genotyping

Thirty-five *Cryptosporidium*-positive samples were genotyped by amplifying a ~830-bp fragment of the small subunit (SSU) rRNA gene by nested PCR (Xiao et al., 2000). After purification, the secondary PCR products were sequenced directly with secondary PCR primers on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence accuracy was confirmed by two-directional sequencing and by sequencing a new PCR product if necessary.

### 2.4. Phylogenetic analysis

Nucleotide sequences were aligned using the ClustalX 1.83 (http://www.clustal.org/), with manual adjustment. Phylogenetic analyses were performed using the software Phylip version 3.67 (http://cmgm.stanford.edu/phylip/). Neighbour-joining trees were constructed based on the evolutionary distances calculated by Kimura-2-parameter model. The reliability of these trees was assessed using the bootstrap analysis with 1000 replicates.

### 2.5. Nucleotide sequence accession number

Representive nucleotide sequences generated in this study were deposited in GenBank database under accession numbers HM116374 to HM116388.

## 3. Results

### 3.1. Prevalence of Cryptosporidium spp

Microscopic analysis of 434 fecal samples showed the presence of *Cryptosporidium* oocysts in 35 samples (8.1%) from 13 of the 31 species of birds (Table 1). *Cryptosporidium* oocysts were found in black-billed magpie (*Pica pica*), Bohemian waxwing (*Bombycilla garrulus*), cockatiel (*Nymphicus hollandicus*), common myna (*Acridotheres tristis*), crested Lark (*Galerida cristata*), fan-tailed pigeon (*Columba livia*), Gouldian finch (*Chloebia gouldiae*), red-billed blue magpie (*Urocissa erythrorhyncha*), red-billed leiothrix (*Leiothrix lutea*), Rufous turtle dove (*Streptopelia orientalis*), silver-eared Mesia (*Leiothrix argentauris*), white Java sparrow (*Padda oryzivora*), zebra finch (*Taeniopygia guttata*). Among these birds with reasonable sample sizes, the highest infection rate (5/9 or 55.6%) was seen in Bohemian waxwing (*Bombycilla garrulus*) and the lowest prevalence (1/21 or 4.8%) in pigeons (*Columba livia domestica* Linnaeus) (Table 1).

### 3.2. Distribution of Cryptosporidium species/genotypes

The SSU rRNA gene of all 35 microscopy-positive samples was successfully amplified by the nested PCR. RFLP analysis of the SSU rRNA gene products and DNA sequencing analysis revealed the presence of five *Cryptosporidium* species/genotypes, including *C. baileyi* (18/35 or 51.4%) in five red-billed leiothrixes (*Leiothrix lu-*

**Table 1**
The prevalence of *Cryptosporidium* species/genotypes in pet birds in Henan, China.

| Host | Sample size | No. of positive samples | Prevalence (%) | *Cryptosporidium* species/genotypes (No. of samples) |
|---|---|---|---|---|
| Black-billed magpie (*Pica pica*) | 1 | 1 | | *C. baileyi* |
| Bohemian waxwing (*Bombycilla garrulus*) | 9 | 5 | 55.6 | *C. meleagridis* (1), *C. galli* (4) |
| Cockatiel (*Nymphicus hollandicus*) | 39 | 8 | 20.5 | Avian genotype V (6), avian geotype III (2) |
| Common myna (*Acridotheres tristis*) | 36 | 4 | 11.1 | *C. baileyi* (4) |
| Crested Lark (*Galerida cristata*) | 9 | 1 | 11.1 | *C. baileyi* (1) |
| Fan-tailed pigeon (*Columba livia*) | 21 | 1 | 4.8 | *C. meleagridis* (1) |
| Gouldian finch (*Chloebia gouldiae*) | 7 | 1 | 14.3 | *C. baileyi* (1) |
| Red-billed blue magpie (*Urocissa erythrorhyncha*) | 1 | 1 | 100 | Avian genotype III (1) |
| Red-billed leiothrix (*Leiothrix lutea*) | 44 | 5 | 11.4 | *C. baileyi* (5) |
| Rufous turtle dove (*Streptopelia orientalis*) | 2 | 1 | 50 | *C. meleagridis* (1) |
| Silver-eared Mesia (*Leiothrix argentauris*) | 7 | 1 | 14.3 | *C. galli* (1) |
| White Java sparrow (*Padda oryzivora*) | 25 | 4 | 16 | *C. baileyi* (4) |
| Zebra finch (*Taeniopygia guttata*) | 40 | 2 | 5 | *C. baileyi* (2) |
| 18 other species of birds | 193 | 0 | 0 | |
| Total | 434 | 35 | 8.1 | *C. baileyi* (18), *C. meleagridis* (3), *C. galli* (5), avian genotype III (3), avian genotype V (6) |